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Original Article

Effect of Aromatase Inhibitor Letrozole on the Placenta of Adult Albino Rats: A Histopathological, Immunohistochemical, and Biochemical Study

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What's Known

- Aromatase inhibitor letrozole has been recently used as the preferred treatment option for ectopic pregnancy.
- To date, no study has investigated the mechanism of letrozole on placental tissue nor its optimal dosage to increase the success rate of an uncomplicated abortion.

What's New

- Letrozole inhibited the expression of estrogen and progesterone receptors in the placenta. It interrupted stimulatory vascular signals, resulting in significant apoptosis and placental vascular dysfunction, facilitating abortion.
- Letrozole in an equivalent human daily dose of 10 mg resulted in a high post-implantation loss rate without imposing severe side effects.

Abstract

Background: Letrozole, an aromatase inhibitor, has recently been introduced as the preferred treatment option for ectopic pregnancy. To date, no study has investigated the effect of letrozole alone on placental tissue. The present study aimed to evaluate the effect of different doses of letrozole on the placenta of rats and to clarify the underlying mechanism.

Methods: Sixty pregnant female rats were equally divided into three groups, namely the control group (GI), low-dose (0.5 mg/Kg/day) letrozole group (GII), which is equivalent to the human daily dose (HED) of 5 mg, and high-dose (1 mg/Kg/day) letrozole group (GIII), equivalent to the HED of 10 mg. Letrozole was administered by oral gavage daily from day 6 to 16 of gestation. Data were analyzed using a one-way analysis of variance followed by Tukey's *post hoc* test and Chi square test. $P<0.05$ was considered statistically significant.

Results: Compared to the GI and GII groups, high-dose letrozole significantly increased embryonic mortality with a high post-implantation loss rate ($P<0.001$) and significantly reduced the number of viable fetuses ($P<0.001$) and placental weight ($P<0.001$) of pregnant rats. Moreover, it significantly reduced placental estrogen receptor (ER) and progesterone receptor (PR) ($P<0.001$) and the expression of vascular endothelial growth factor ($P<0.001$), while increasing the apoptotic index of cleaved caspase-3 ($P<0.001$).

Conclusion: Letrozole inhibited the expression of ER and PR in rat placenta. It interrupted stimulatory vascular signals causing significant apoptosis and placental vascular dysfunction. Letrozole in an equivalent human daily dose of 10 mg caused a high post-implantation loss rate without imposing severe side effects.

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Keywords ● Letrozole ● Placenta ● Receptors ● Estrogen ● Progesterone ● Vascular endothelial growth factor ● Caspase

Introduction

Ectopic pregnancy (EP) is the implantation of an embryo outside the uterine cavity. EP is the leading cause of maternal morbidity and mortality, and approximately 1-2% of all pregnancies are ectopic.[1] Despite the declining incidence of EP-related mortality, ruptured EP accounts for approximately 6% of all maternal deaths. Some

EPs resolve spontaneously. However, medical or surgical management is required when an EP continues to grow.[2] If an EP is diagnosed early, treatment with methotrexate is recommended in many cases. Methotrexate is primarily a chemotherapeutic agent for cancer treatment and has adverse effects on various body systems.[3] As an alternative, letrozole is recommended due to its cost-effectiveness and safety.

Letrozole, an aromatase inhibitor, is certified for the treatment of estrogen-sensitive breast cancer. It inhibits estrogen production through peripheral aromatization of androgen by suppressing estrogen biosynthesis enzymes such as cytochrome P450 aromatase.[4] Due to its high tolerability, low cost, and low side effects, letrozole is also used to treat many gynecological disorders, e.g., to reduce uterine myomas, induce ovulation in cases of polycystic ovarian syndrome, and prevent recurrence of endometriosis.[5] Letrozole inhibits estrogen production during pregnancy and negatively affects placental development, progesterone levels, and the expression of vascular endothelial growth factor (VEGF). It subsequently disrupts the physiological function of progesterone, which is essential during the early stages of pregnancy.[6] Interruption of VEGF signaling, an angiogenic factor responsible for the implantation and placentation of EP in the oviduct,[7] could be the mechanism by which letrozole inhibits the progression of pregnancy.

Quantitative beta-human chorionic gonadotropin (β-HCG) measurement is the best and most straightforward method to monitor the progression of early pregnancy. However, there is a controversy over the presence of placental gonadotropin in rodents.[8] A previous study evaluated the use of letrozole combined with methotrexate for the treatment of EP and medical management of first-trimester missed miscarriage.[9] They reported the effectiveness of letrozole followed by misoprostol in the management of first-trimester missed miscarriage. Mitwally and colleagues stated that the role of estrogen in early pregnancy were underestimated.[10] It is also suggested that inhibition of estrogen production with letrozole alone could affect physiological signals essential for the progression and maintenance of pregnancy.[10] Due to several side effects and risks of methotrexate, Mitwally and colleagues evaluated the effect of letrozole 5 mg daily for 10 days and reported relatively low pregnancy termination rates. However, compared to the use of methotrexate, there are still controversies regarding the use of letrozole and its optimal dosage.

Given the above and to elucidate the underlying mechanism, the present study aimed to evaluate the effect of different doses of letrozole on the placental structure and post-implantation loss rate. In addition, we investigated the optimal and safe dose of letrozole for the termination of early pregnancy.

Materials and Methods

Animals

Adult male and nulliparous female Wister albino rats were obtained from the Animal House of Tanta University (Tanta, Egypt). The animals were kept in clean cages under standard laboratory conditions regarding temperature, humidity, and light/dark cycles. Throughout the experiment, the animals had free access to water and a standard laboratory diet. The composition of the standard diet included 23.5% protein, 48.8% carbohydrate, 5% lipid, 12% water, 5% ash, 5% cellulose, and 0.7% vitamin and mineral mixture. After one week, females were mated to male rats in a 1: 1 ratio during the dark cycle. At the end of the dark cycle (8:00 AM), the presence of sperm in the vaginal smears was taken as day zero of gestation.

The study was conducted in accordance with the recommendations for the treatment and use of animals of the National Institutes of Health (NIH), publication number 8023, 1978 revision. The study was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine of both Tanta (approval number: 35895) and Zagazig (approval number: ZU-IACUC/3/F/86/21) Universities in Egypt.

Animal Experiments

Letrozole 2.5 mg tablets were purchased from Novartis Pharma AG (Basel, Switzerland). Sixty pregnant female rats were equally divided into three groups, namely the control group (GI), low-dose letrozole group (GII), and high-dose letrozole group (GIII). Low-dose letrozole (0.5 mg/Kg/day) is a human equivalent dose (HED) of 5 mg, and high-dose (1 mg/Kg/day) is HED of 10 mg. All rats were studied from day 6 to 16 of gestation, which is approximately equivalent to day 12 to 52 of gestation in humans.[11] Doses were calculated based on body surface area conversion from humans to rats.[12]

The animals were monitored daily for water consumption, general status, and clinical signs of toxicity. Rats' body weight was measured daily before letrozole was administered, and the dose was adjusted accordingly. Pregnancy was terminated on day 16 of gestation by sacrificing rats using 1.9% inhaled diethyl ether (0.08 mL/Liter of container volume). Blood samples were

collected to measure blood urea nitrogen (BUN), serum creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), estradiol and progesterone levels, as well as for platelet count. In addition, maternal weight, absolute maternal weight, fetal weight, early and late resorptions, and the number of live and dead fetuses were also noted. The placenta of each rat was collected, weighed, and cut at the insertion of the umbilical cord in an identical binary manner. Half of the samples were prepared for light microscopic studies, and the remainder were used for tissue homogenate. Given the controversy regarding the presence of placental gonadotropin in rodents,[13] serum progesterone levels were used to monitor pregnancy progression instead of β-HCG.

Light Microscopy Examination

The placentas were cut into 5 mm slices and fixed in formaldehyde buffer solution (10%), gradually dehydrated by scaling concentrations of ethyl alcohol, cleared with xylene, and embedded in paraffin wax.[14] Sequential five microns thick slices were used to study histopathological changes in the rat placenta using hematoxylin and eosin (H&E) staining. In addition, immunohistochemical staining for primary antibodies was performed using a BOND-MAXTM stainer (Leica Biosystems Nussloch GmbH, Nussloch, Germany). Primary antibodies were anti-VEGF monoclonal antibody (catalog number: MA1-16629) and cleaved caspase-3 (catalog number: MA1-16629), both from Invitrogen ThermoFisher Scientific, USA. The placental tissue of the control group was used as a positive control for VEGF and the appendix tissue as a positive control for cleaved caspase-3, while omitting the primary antibody and using the secondary antibody alone as a negative control.

Quantitative Real-time Polymerase Chain Reaction

The expression of estrogen receptor (ER) and progesterone receptor (PR) mRNA in rat placenta was examined using reverse transcription polymerase chain reaction (RT-PCR). Whole RNA was isolated from the tissue homogenate, and cDNA was produced using one-step RT-PCR (QIAGEN Sciences, Germantown, Maryland, USA) according to the manufacturer's instructions. RT-qPCR was performed using the Mx3005P® system (Stratagene, La Jolla, CA, USA) with 2x QuantiTect SYBR Green Master Mix Green (QIAGEN, USA). Denaturation of the reaction mixture was performed at 95 °C for five min, amplified in 35 cycles at 95 °C for 15 sec, annealing at 62 °C for 30 sec, and extension at 72 °C for 20 sec. β-actin primer was used to normalize the data, and the $2^{-\Delta\Delta CT}$ method was used to calculate gene expression levels. The sequence of primers used is presented in table 1.

Morphometric Analysis

Ten different non-overlying randomly chosen fields from each slide were observed for quantitative measurements. The mean thickness (in μm) of the labyrinth and basal regions was measured in H&E-stained slices at 40× amplification from the point of convexity, while the average of the long and short axes was used for the irregularly formed placenta. The number of positive cells for cleaved caspase-3 was determined by counting 10 fields in the labyrinth and basal regions with a 40× objective. VEGF expression of immune cells was determined by multiplying color intensity with the percentage of mean area. Color intensity was defined as weak, moderate, or strong in 3'3-diaminobenzidine tetrahydrochloride hydrate stained slides under 400× amplification.

Statistical Analysis

Data were analyzed using SPSS software version 25.0 (IBM Corp., Armonk, NY, USA) and expressed as mean±SD. Continuous variables were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The Chi square test was used to assess the difference between the mean of categorical variables. $P<0.05$ was considered statistically significant.

Results

Of the 60 pregnant rats, 20 (GII) received a daily low-dose letrozole (0.5 mg/Kg/day, HED: 5 mg), 20 (GIII) a high-dose letrozole (1 mg/Kg/day,

Table 1: The sequence of estrogen receptor, progesterone receptor, and β-actin primers used for the real-time polymerase chain reaction

Name	Primer Sequence (5'→3')
Estrogen receptor	Forward: 5′-ATGAAAGGTGGGATACGAAAAGACC-3′ Reverse: 5′-TGCCAGGTTGGTCAGTAAGCC-3′
Progesterone receptor	Forward: 5′-CGCGCTCTACCCTGCACTC-3′ Reverse: 5′-TGA ATCCGGCCTCAGGTAGTT-3′
β-actin	Forward: 5′-ACGATGCCCCCCGGGCCGTCTT-3′ Reverse: 5′-TCTCTTGCTCTGGGCCTCGTCGCCC-3′

HED: 10 mg), and the remaining rats (n=20) were used as controls (GI). The effect of different doses of letrozole on various maternal and fetal parameters is presented in table 2.

There was a significant increase in embryonic mortality with a high post-implantation loss in the GIII group (171±12) compared to the GI (13±2) and GII (109±6) groups (P<0.001). Embryonic death during the post-implantation period significantly increased in the GIII group (74.8%) compared to the

Table 2: The effect of different doses of letrozole on various maternal and fetal parameters

Variables		Group type			P value*	Multiple comparisons**		
		Group I (n=20)	Group II (n=20)	Group III (n=20)		GI and GII	GI and GIII	GII and GIII
Maternal body weight (g) at day 19	Mean±SD	380.435±7.754	315.760±5.450	295.870±8.423	<0.001	<0.001	<0.001	<0.001
	Range	368.9-391.4	325.9-310.9	285.5-307.7				
Maternal absolute weight (g)	Mean±SD	269.190±5.711	271.200±3.560	270.315±4.901	0.18			
	Range	256.9-275.1	263.7-279.5	266.8-275.9				
No. of animals at term with live fetuses (n=20)	n (%)	20 (100%)	15 (75%)	9 (45%)	<0.001	0.047	<0.001	0.053
Implantations	Mean±SD	201.550±7.708	235.350±5.815	230.900±5.495	<0.001	<0.001	<0.001	0.081
	Range	189-216	220-249	222-240				
Post-implantation loss	Mean±SD	13±2	109±6	171±12	<0.001	0.001	0.001	0.001
	Range	10-16	100-120	152-192				
No. of implantations per litter	Mean±SD	14.150±1.725	13.00±1.298	13.700±1.625	0.072			
	Range	11-17	11-15	10-16				
Post-implantation lose rate	Mean±SD	6.5±0.4	46.8±1.2	74.8±1.8	<0.001	<0.001	<0.001	<0.001
No. of viable fetuses per litter	Mean±SD	13.7±1.257	6.750±1.118	3.250±0.786	<0.001	<0.001	<0.001	<0.001
	Range	11-15	5-9	2-4				
Fetal weight	Mean±SD	3.71±0.2	3.53±0.1	3.35±0.2	<0.001	<0.001	<0.001	<0.001
	Range	3.44-3.92	3.40-3.71	3.08-3.62				
Placental weight	Mean±SD	0.601±0.004	0.465±0.003	0.217±0.001	<0.001	<0.001	<0.001	<0.001
	Range	0.59-0.61	0.46-0.475	0.21-0.227				
Placental labyrinth zone thickness	Mean±SD	460.56±29.21	179.28-22.54	116.28±12.34	<0.001	<0.001	<0.001	<0.001
	Range	410.2-498.8	146.54-213.87	102.12-130.67				
Placental basal zone thickness	Mean±SD	127.48±16.72	55.08±9.97	25.12±8.78	<0.001	<0.001	<0.001	<0.001
	Range	103.67-145.76	34.65-70.43	37.23-15.89				
Maternal serum progesterone (ng/mL)	Mean±SD	36.175±1.23	18.435±1.118	11.425±1.132	<0.001	<0.001	<0.001	<0.001
	Range	34.3-38.7	16.2-20.1	9.6-13.7				
Maternal serum estradiol (pg/mL)	Mean±SD	58.18±5.82	19.65±1.7	16.22±0.92	<0.001	<0.001	<0.001	<0.001
	Range	50.3-66.8	17.40-21.17	17.83-14.92				
Maternal serum creatinine (mg/dL)	Mean±SD	0.49±0.17	0.51±0.12	0.45±0.15	0.43			
	Range	0.25-0.72	0.37-0.69	0.27-0.59				
Maternal blood urea nitrogen (mg/dL)	Mean±SD	23±6	24±7	22±5	0.57			
	Range	16-31	17-32	16-28				
Maternal ALT level (U/L)	Mean±SD	22.63±0.97	31.690±1.367	77.330±2.900	<0.001	<0.001	<0.001	<0.001
	Range	21.2-24.1	29.6-35.5	69.4-80.1				
Maternal AST level (U/L)	Mean±SD	34.425±1.778	41.160±1.898	93.065±4.402	<0.001	<0.001	<0.001	<0.001
	Range	29.9-37.9	35.9-43.9	80.8-99.7				
Maternal platelets ($\times 10^4/\mu L$)	Mean±SD	128±9	126±5	129±7	0.39			
	Range	117-137	119-133	120-131				
Placental ER	Mean±SD	1.1±0.08	0.68±0.09	0.56±0.11	<0.001	<0.001	<0.001	<0.001
	Range	0.91-1.15	0.88-1.14	0.82-1.13				
Placental PR	Mean±SD	1.09±0.09	0.69±0.06	0.51±0.11	<0.001	<0.001	<0.001	<0.001
	Range	0.96-1.17	0.61-0.79	0.23-0.59				
Placental caspase-3C	Mean±SD	10±2	25±7	39±4	<0.001	<0.001	<0.001	<0.001
	Range	7-13	17-34	32-45				
Placental VEGF	Mean±SD	121.825±9.585	64.340±2.509	22.340±1.473	<0.001	<0.001	<0.001	<0.001
	Range	32.2-45.4	29.8-34.6	18.8-25.4				

*ANOVA: One-way analysis of variance; **Chi square test; Mean±SD: Mean±standard deviation; GI: Control group; GII: Low dose letrozole (0.5 mg/Kg/day) group; GIII: High dose letrozole (1 mg/Kg/day) group; Bun: Blood urea nitrogen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase (AST); ER: Estrogen receptor; PR: Progesterone receptor; VEGF: Vascular endothelial growth factor. All body weights at term minus the gravid uterine weight, post-implantation loss includes early/late resorptions and dead fetuses, post-implantation loss rate calculated by dividing the mean number of post-implantation loss by the mean number of implantations ×100.

GI (6.5%) and GII (46.8%) groups (P<0.001). The number of viable fetuses per litter and placental weight in the GIII group were significantly lower than the other two groups (P<0.001).

The mean placental weight was significantly lower in the GIII group (0.217±0.001 g) compared to the GI (0.601±0.004 g) and GII (0.465±0.003 g) groups (P<0.001) (table 2, figure 1). The results of placental thickness analysis showed that the mean thickness of the labyrinth and basal regions were significantly lower in the GIII group (116.28±12.34 and 25.12±8.78 µm, respectively) compared to the GI (460.56±29.21 and 127.48±16.72 µm, respectively) and GII (179.28±22.54 and 55.08±9.97 µm, respectively) groups (P<0.001) (table 2, figure 2)

Histopathological Results

The H&E stained slides of the rats in the GI group showed a normal placenta structure composed of three morphological areas, namely basal, decidua, and labyrinth. The labyrinth formed a web-shaped vascular network between maternal blood sinuses and capillary networks for fetal blood circulation. Both circulations were strongly interdigitated. However, they were compartmentalized via the interhaemal membrane consisting of syncytiotrophoblast and cytotrophoblast cells. Fetal capillaries were lined with endothelial nuclei and had a smaller luminal width than the maternal sinuses. The basal area mainly consisted of spongiotrophoblast cells with basophilic cytoplasm intermixed with glycogen trophoblast cells, which showed clear and vacuolated cytoplasmic characteristics. Large trophoblastic cells with euchromatic nuclei were found at the margin between the decidua and the basal area (figures 3a and 3b). In contrast, the placental tissues of the letrozole-treated groups showed a significant reduction in the width of the basal and labyrinth areas (figures 2b and 2c). The letrozole-treated groups also showed marked degenerative changes in the basal layer of the placenta in the form of congestion, hemorrhage, and lysis of glycogen trophoblast cells, leaving areas filled with eosinophilic fluid (figure 3c), cystic degenerative changes in the basal layer (figure 3d), and marked trophoblastic apoptosis (figure 3f).

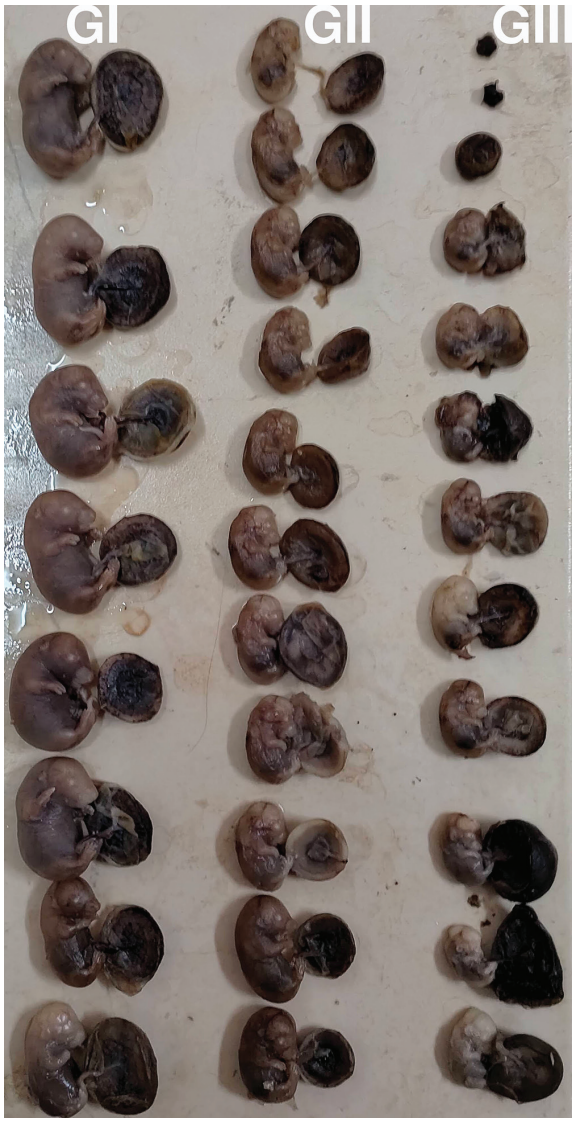

Figure 1: Morphological changes in fetus and placenta of the letrozole-treated groups compared to the control group. GI: Control group; GII: Low-dose letrozole group; GIII: High-dose letrozole group

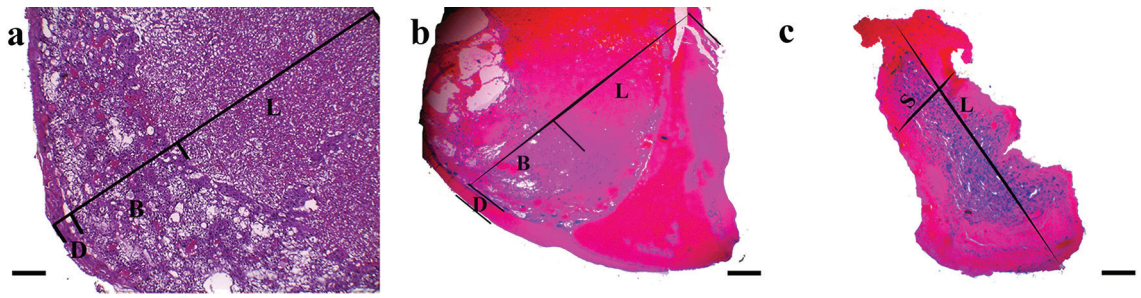

Figure 2: Photomicrographs of hematoxylin and eosin-stained placental sections of the control and letrozole-treated groups. **a**: Normal architecture of rat placenta in the control group consisting of labyrinth zone (L), basal zone (B), and decidual zone (D). **b**: Degenerative changes of rat placenta in the low-dose letrozole group consisting of labyrinth zone (L), basal zone (B), and decidual zone(D). **c**: Degenerative placenta with irregular diameter in the high-dose letrozole group, where (L) shows the long axis and (S) refers to the short axis. Scale bars=200 µm.

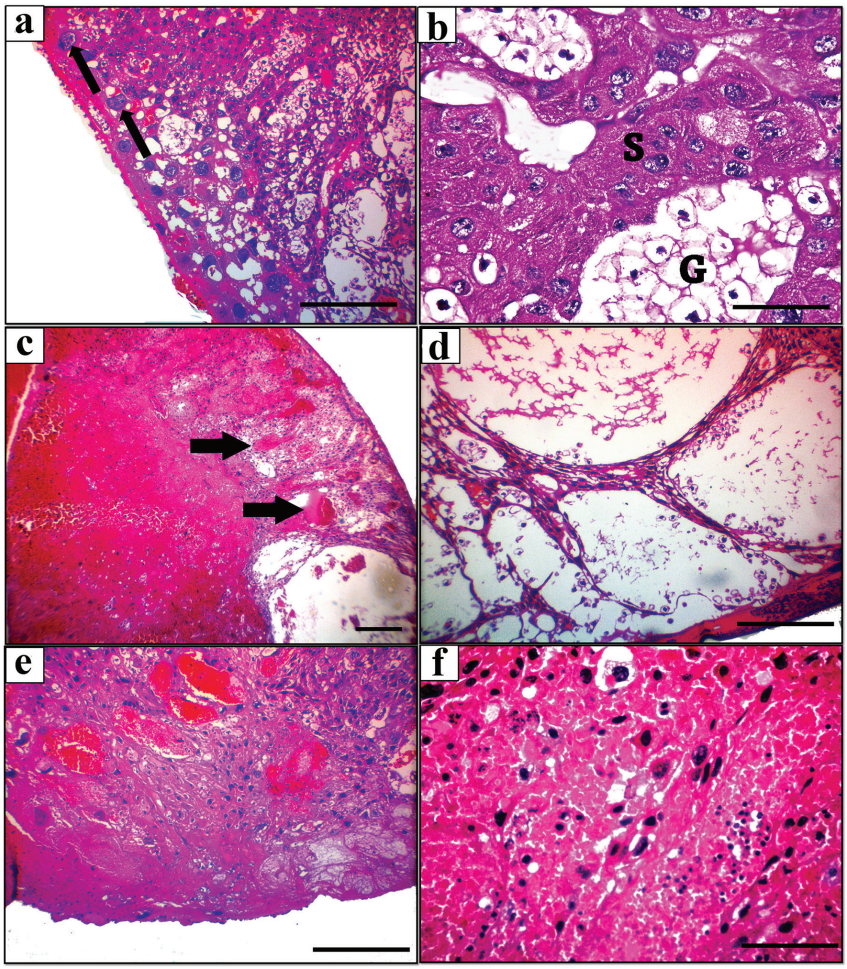

Figure 3: Photomicrographs of hematoxylin and eosin-stained placental sections show degenerative changes in the basal layer of the placenta of the letrozole-treated groups compared to the control group. a and b: The basal layer of the control group shows a normal basal zone consisting of spongiotrophoblast cells (S) with basophilic cytoplasm intermixed with glycogen cells (G) showing clear vacuolated cytoplasm, and giant trophoblast cells (arrows) with large euchromatic nuclei (scale bar=100 µm). c: The basal layer of the letrozole-treated groups shows congestion, hemorrhage, and eosinophilic fluid (arrow) (scale bar=200 µm). d: Cystic degenerative changes in the basal layer (scale bar=100 µm). e: Apoptotic trophoblastic cells and dilated congested blood vessels (scale bar=100 µm). f: Apoptotic trophoblastic cells and eosinophilic material in the basal layer (scale bar=100 µm).

The labyrinth area also showed the rupture of the interhemal membrane and loss of blood compartmentalization with marked hemorrhage and degenerative disruption of the vascular network (figure 2b). The cytotrophoblasts and endothelial cells also showed dark-stained apoptotic nuclei alongside cystic spaces filled with eosinophilic fluid (figures 4b to 4d).

Immunohistochemical Results

Cleaved caspase-3: Low expression levels of cleaved caspase-3 were observed in the GI group (figures 5a and 5b) compared to the letrozole-treated groups with several positive cells (figures 5c and 5d). Morphometric examination of the mean value of cleaved caspase-3 positive cells showed a significant increase in the GIII (39±4) group compared to the GI (10±2) and the GII (25±7) groups (P<0.001) (table 2).

VEGF: The placental sections of the GI group showed high cytoplasmic VEGF expression in the trophoblasts of the labyrinth and basal area (figures 6a and 6c). However, the letrozole-treated groups showed focal areas with weak cytoplasmic VEGF expression in the labyrinth and basal areas (figures 6b and 6d). Morphometrical and statistical analysis showed a significantly lower VEGF expression of immune cells in the GIII group (22.34±1.47) compared to the GI (121.825±9.585) and GII (64.340±2.509) groups (P<0.001).

Quantitative Real-time PCR: The results of the RT-qPCR test showed a markedly significant decrease in the ER and PR mRNA expression levels in the GIII group (0.56±0.11 and 0.51±0.11, respectively) compared to the GI (1.1±0.08 and 1.09±0.09, respectively) and GII (0.68±0.09 and 0.69±0.06, respectively) groups (P<0.001).

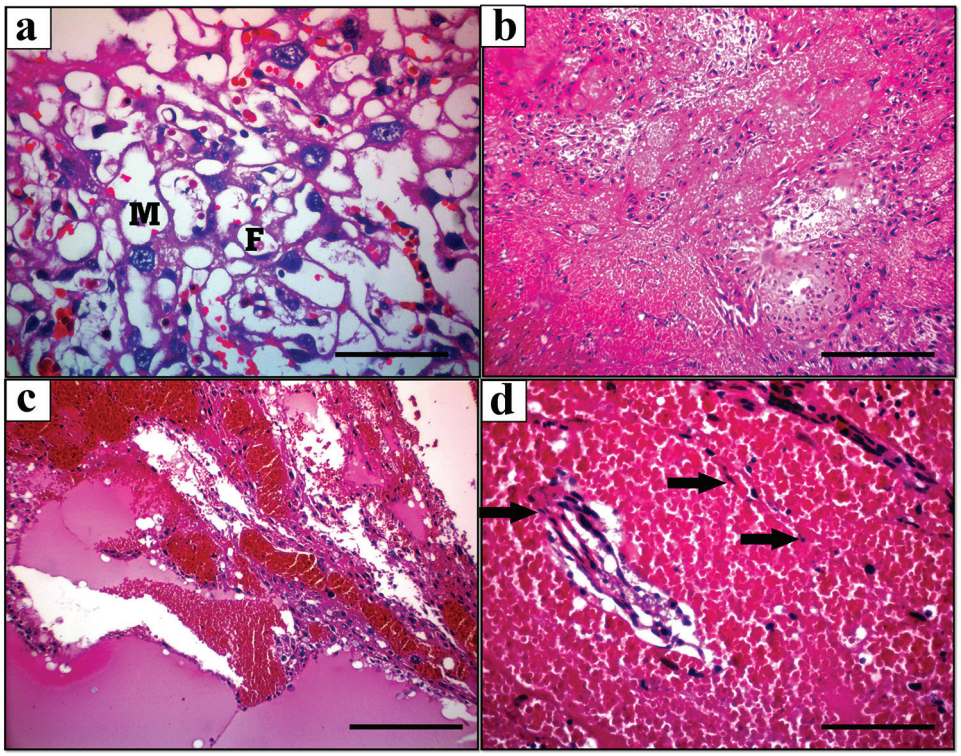

Figure 4: Photomicrographs of hematoxylin and eosin-stained placental sections showing degenerative changes of the labyrinth layer of the placenta in the letrozole-treated groups compared to the control group. a: The labyrinth layer of the control group shows a normal labyrinth composed of a web-shaped vascular network between maternal blood sinuses (M) and fetal capillaries (F) separated by an interhemal membrane containing cytotrophoblast and syncytiotrophoblast cells. b: The labyrinth layer of the letrozole-treated groups shows a loss of compartmentalization with the accumulation of eosinophilic materials. c: Congestion, hemorrhage, and eosinophilic fluid-filled spaces. d: Apoptosis of the cytotrophoblasts and endothelial cells (arrow). Scale bars=100 µm.

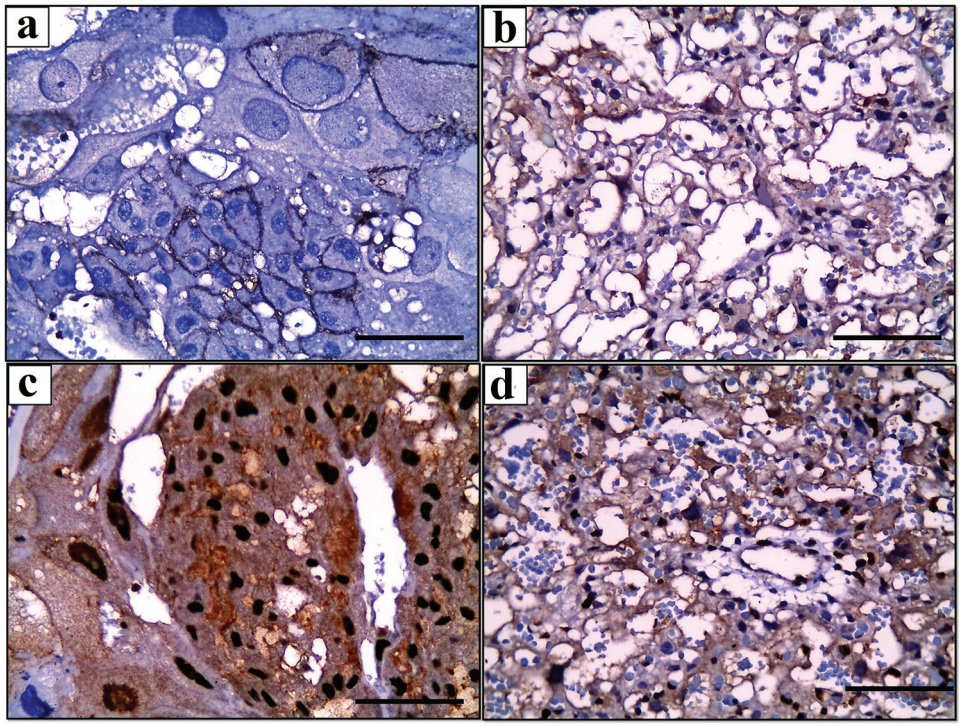

Figure 5: Photomicrographs of cleaved caspase-3. Immunohistochemistry shows a high placenta apoptotic index in the letrozole-treated groups compared to the control group. a: Low expression of cleaved caspase-3 in the basal layer of the control group. b: Low expression cleaved caspase-3 of the labyrinth layer of the control group. c: High expression of cleaved caspase-3 in the basal layer of the letrozole-treated groups. d: High expression of cleaved caspase-3 in the labyrinth layer of the letrozole-treated groups. Scale bars=100 µm.

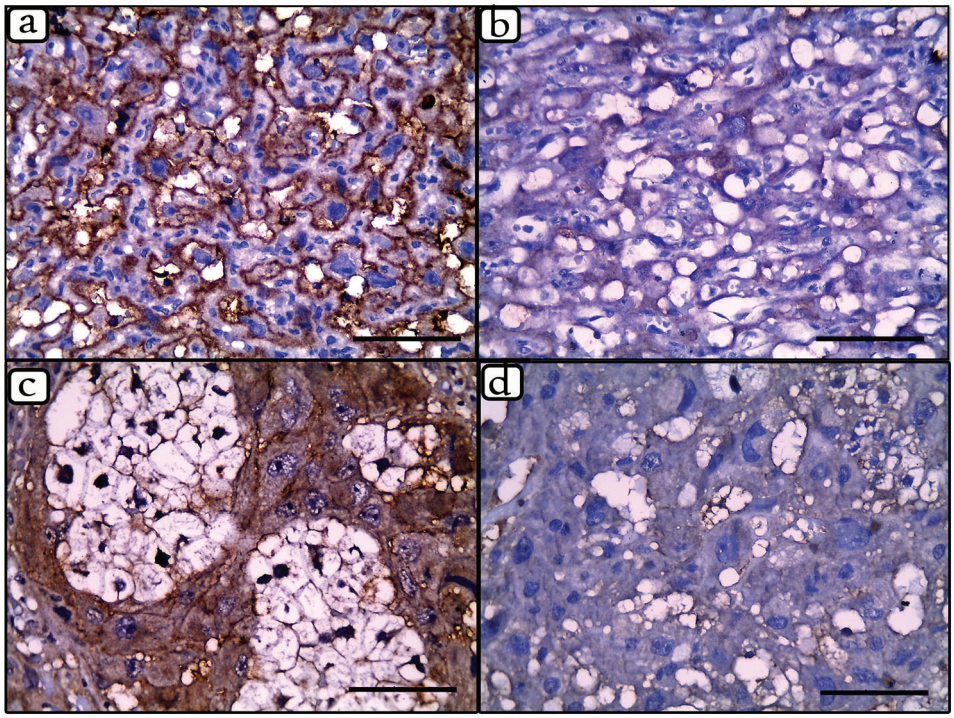

Figure 6: Photomicrographs of VEGF. Immunohistochemistry shows high expression in the control group compared to the letrozole-treated groups. a: Strong expression of VEGF in the labyrinth layer of the control group. b: Faint expression of VEGF in the labyrinth layer of the letrozole-treated groups. c: High cytoplasmic expression of VEGF in the cells of the basal layer of the letrozole-treated groups. d: Low expression of VEGF in the basal layer of the letrozole-treated groups. Scale bars=100 µm.

Biochemical Results

There was no statistically significant difference in serum creatinine and BUN levels, and platelet count between the three groups. However, there was a significant increase in the serum ALT and AST levels in the GIII group (77.33±2.90 and 93.06±4.40 U/L, respectively) compared to the GI (21.2±0.97 and 34.42±1.77 U/L, respectively) and GII (31.69±1.36 and 41.16±1.89 U/L, respectively) groups (P<0.001) (table 2). Furthermore, there were significantly lower levels of short-term progesterone in the GIII group (11.42±1.13 U/L) than the GI (36.17±1.23 U/L) and GII (18.43±1.11 U/L) groups (P<0.001). In terms of serum estradiol, the GIII group showed significantly lower levels (16.22±0.92 U/L) than the GII (19.65±1.7 U/L) group (P<0.001).

Discussion

Similar to previous studies, low-dose (0.5 mg/Kg/day, HED: 5 mg) and high-dose (1 mg/Kg/day, HED: 1 mg) letrozole were used.[10, 16] In line with a study by Tiboni and colleagues,[4] our results showed that the administration of high-dose letrozole significantly increased embryonic mortality associated with post-implantation loss and reduced the number of viable fetuses. ER and PR expressions play a crucial role in maintaining pregnancy. Similar to our findings, Lee and colleagues reported that letrozole significantly reduced the expression of ER and PR in rat placenta.[3] Low estrogen levels due to letrozole blocking PRs and subsequently disrupting physiological functions of progesterone are necessary to maintain early pregnancy.[15]

VEGF is a potent mitogenic factor that promotes angiogenesis, and blood vessel development in the fetoplacental unit and is necessary to support fetal growth.[16] Our results showed that exposure of rat placenta to a high dose of letrozole caused a significant reduction in VEGF. Excessive angiogenesis is the main factor involved in implantation and placentation, which are critical early processes in the development of a viable pregnancy. The production of VEGF is increased in hypoxic conditions, such as the implantation environment in the oviduct, resulting in the development of more active angiogenesis.[17] Therefore, targeting VEGF and its stimulators is the most effective approach to terminate EP. Estrogen is one of the major stimulative regulatory factors of VEGF, supporting capillary growth and permeability in the uterus. However, the administration of antiestrogenic drugs inhibits angiogenesis and can cause a complete failure of embryo expansion due to interference with placenta formation and embryonic vascular development.[18]

Cell death, or apoptosis, is usually present in

tissue development (e.g., placental tissues) and during cell growth. Increased placental apoptosis is found in pre-eclampsia and intrauterine growth restriction. A high apoptotic index in the placenta can reduce fetal and placental weight.[19] Caspase-3, one of the proteases, plays an important role in initiating and executing the apoptosis cascade. In our study, based on the expression levels of cleaved caspase-3, high-dose letrozole showed to significantly increase significantly increase the placental apoptotic index.

Progesterone is one of the main hormones that play an important role in fetal development. It is known as the "pregnancy hormone", as it increases progressively at each stage of pregnancy. In rats, it gradually reaches its peak level (36 ng) on day 19 of the third trimester, while this level in non-pregnant female rats is 5 ng. A decrease in progesterone levels in different trimesters indicates reduced viability of a pregnancy.[20] Our results showed that progesterone levels were significantly reduced in the high- and low-dose letrozole groups compared to the control group. Moreover, the effect of high-dose letrozole in reducing progesterone levels was significantly higher than low-dose letrozole. A significant increase in hepatic enzymes (ALT and AST) was also found in rats given high-dose letrozole. Gharia and colleagues also reported an increase in the liver function parameters of female rats treated with an equivalent dose of letrozole.[21] Letrozole is associated with elevated levels of liver enzymes in up to 1% of women treated with low doses over a long period. The increase is generally asymptomatic and self-limiting, processed by the cytochrome P450 system in the liver, and rarely requires dose adjustment.

Termination of pregnancy and fetal loss due to letrozole can be attributed to the absence of estrogen-related growth signals to the placenta and fetus. Letrozole affects fetus development and growth due to its potential teratogenic effects that cause severe malformation and disruption of organogenesis. These in turn undermine continued pregnancy since estrogen deficiency impairs fetal development.[4] Although the rat placenta does not produce estrogen, placental development is estrogen-dependent, since estrogen signals are associated with the proliferation and differentiation of placental trophoblasts.[22] In addition, the rat placenta is the primary source of testosterone in the peripheral circulation during pregnancy. Testosterone serves as a substrate for estradiol synthesis in the corpus luteum, thus maintaining ovarian estradiol production.[23]

It is also worth noting the differences in angiogenesis between intrauterine and ectopic pregnancies. Placental growth factor (PIGF) is a proangiogenic protein, with similarities to VEGF, secreted by the local decidual environment in normal intrauterine pregnancy. PIGF acts through VEGF receptors to facilitate angiogenesis and the development of a supportive vascular network.[24] In EP, however, maternal cells at the implantation site often exhibit limited decidual cell differentiation. When present, these cells significantly reduce PIGF secretion, affect the environment, and are associated with tissue hypoxia. This in turn adds a further inhibition of PIGF expression in EP.[25] As compensation, in response to hypoxia and angiogenic stimulators (e.g., estrogen), ectopic trophoblastic tissue increases the production of VEGF more than the normal intrauterine trophoblastic tissue, allowing it to develop further. While tubal implantations that cannot overcome these adverse conditions resolve spontaneously.[26]

In tubal pregnancies, implantation and early placental development processes occur in the same way as in the uterus. Furthermore, extravillous cytotrophoblast cells invade the tubal vessels similar to the spiral arteries in the uterus. Therefore, we investigated the exposure of rat placenta to letrozole from day six of gestation. This corresponds to days 11 and 12 of gestation in humans, which is approximately the time of EP development and detection.[27]

Both pharmaceutical and surgical methods can be used to manage the growth of EP. Medical management overrides surgical methods since surgical abortion is invasive and puts a huge financial burden on the patients and the healthcare system. It is also associated with several side effects, including failure to remove trophoblasts, injuries to the bowel and major blood vessels, hemorrhage, sepsis, and death. However, these side effects are more likely to occur if abortion is not performed in a well-equipped hospital under the supervision of a healthcare professional.[28] Given the potential and safety of letrozole compared to other drugs (e.g., methotrexate), further studies are recommended to evaluate the use of letrozole alone as a medical treatment for EP.[5, 15]

Conclusion

Letrozole inhibited the expression of ER and PR in the rat placenta. It interrupted stimulatory vascular signals, causing significant apoptosis and placental vascular dysfunction, and facilitating abortion. Furthermore, letrozole in an equivalent human daily dose of 10 mg caused a significantly high post-implantation loss rate without imposing severe side effects.

Authors' Contribution

All authors contributed equally to the study design, data analysis and interpretation, and drafting and revision of the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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